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Antiproliferative effect of green synthesised silver, copper and bimetallic Ag-Cu nanoparticles using *Achyranthes aspera* against human breast adenocarcinoma cancer cells *in vitro*.

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The green synthesis of metal nanoparticles utilising plant extract is an environmentally sustainable and economical approach for their production. The present study reports the synthesis of metallic nanoparticles (NPs) AgNPs, CuNPs, and bimetallic Ag-CuNPs using aqueous stem extract of *Achyranthes aspera* and characterised using UV-Visible spectroscopy, Fourier Transform Infra-Red (FTIR) spectra, Scanning Electron Microscopy (SEM), and X-ray diffraction (XRD) methods. The UV-Vis spectrum of the bimetallic Ag-CuNPs shows a strong peak at 400nm, and secondary peak at 540nm indicates the formation of nanoparticles. FTIR spectrum confirms the presence of phenolic groups, flavonoids, and alkaloids, which play a critical role in the reduction and stabilising the nanoparticles. SEM analysis revealed the bimetallic Ag-CuNPs exhibited both rod-like and spherical morphologies with size ranges from 124 to 198 nm. The XRD pattern showed characteristic peaks of 2θ at 16.51º, 20.92º, 26.68º, and 39.5º and corresponding crystal planes (111), (200), (220), and (400), indicating the face-centered cubic (FCC) and crystalline structure of the biosynthesised bimetallic Ag-CuNPs. Furthermore, *in vitro* cytotoxicity assays against MCF-7 breast cancer cell lines revealed a potent antiproliferative effect, with an IC_{50} value of 55.28 ± 0.517 µg/mL, highlighting the therapeutic potential of Ag-Cu bimetallic nanoparticles.

Keywords: Anticancer activity, AgNPs, CuNPs, Bimetallic Ag-CuNPs, MCF-7, MTT assay

Cancer is among the most lethal disease affecting humans; it occurs when aberrant cells proliferate out of control, invading neighboring tissues and causing disruptions. *According to the WHO, breast cancer is the most common cancer diagnosed in women* and the second most common cause of death from cancer among women worldwide, accounting for 20 million new cases and 9.7 million deaths worldwide. Out of which 1 in 9 men and 1 in 12 women are expected to die from cancer. Cisplatin and its analogs were utilised to treat a variety of cancers in clinical therapies such as radiation, chemotherapy, immunomodulation, and surgery, which resulted in serious adverse effects like ototoxicity and neurotoxicity. Furthermore, cisplatin resistance is increasingly developing in certain tumor cell lines. Thus, scientists are working to create novel substances to fight different types of cancer and reduce the side effects of current treatments[1-4]. Conventional methods for creating nanoparticles have proven challenging, due to their high cost, time-consuming nature, high chemical dosages, hazardous byproducts, and low yields. Because of environmental concerns, researchers are working to replace nanoparticles with green synthesis using plant extract and microorganisms due to their biocompatibility[5,6].

One choice while researching innovative cancer treatment strategies is the use of nanoparticles derived from medicinal plants considered as the best source of anticancer medications[6], because of their anti-mutagenic and antioxidant properties. For more than 30 years, pharmacological carriers in the form of nonmaterial have been used to boost the *in vivo* anticancer action of drugs. There are numerous natural chemical compounds present in the plants called phytochemical compound, which are biologically active includes terpenoids, phenols, lignans, tannins, flavonoids, quinones, coumarins, and alkaloids, which, inhibits cell cycle checkpoints, promote apoptosis by activating initiator and executor caspase and have significant role as antioxidant, anti-inflammatory, antitumor, antimutagenic, and anti-carcinogenic properties[2,7-10]. Since many years, a Perennial herb belonging to Amaranthaceae family has been used as a medicine, because stems, roots, and leaves have therapeutic qualities that help treat a variety of illnesses, including Antimicrobial, Antiparasitic, and Anticancer effects [11,12,] Spermicidal[13], Neuroprotective[14], Gastroprotective[15], Wound healing[16] Hepatoprotective[17]. In this report, the *Achyranthes aspera* aqueous stem extract was utilised for the preparation of AgNPs, CuNPs, and bimetallic Ag–Cu nanoparticles (NPs) and their antiproliferative activity by MTT assay in MCF-7 cancer cell line.

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Materials and Methods

Chemicals

All chemicals were purchased from Himedia and Sigma Aldrich- Silver nitrate ($AgNO_3$), Copper sulfate solution ($CuSO_4$. $5H_2O$), NaOH, MTT reagent. Stem of *Achyranthes aspera* were collected within the premises of Ghatkesar

Preparation of *Achyranthes aspera* stem extract

Achyranthes aspera stem was thoroughly cleaned under running tap water and then deionized water to eliminate any remaining dust particles from its surface. Then stems were shade-dried and ground into fine powder. 25g of powdered stem was boiled in a 250mL of double distilled water for 20 minutes at 60°C while stirring occasionally. The mixture is cooled to room temperature and filtered using Whatman no.1 filter paper and stored at 4°C for further experimental analysis[18].

Synthesis of AgNPs and CuNPs

The AgNP is synthesised using 10mL of aqueous stem extract and added to 90mLl of 1mM $AgNO_3$ solution, followed by heating at 80°C for 3 hours with constant stirring. The change in the colour from yellow to brown indicates the formation of silver nanoparticle[19]. For synthesis of *A. aspera* copper nanoparticles, 50mL of 5mM copper sulfate solution was mixed with 5 mL of aqueous stem extracts. The pH value 7.0 adjusted for the mixture by the addition of NaOH (1 N) solution. Further, the green colour mixture was obtained indicates the formation of copper nanoparticle[20].

Synthesis of bimetallic Ag-CuNPs

The equal proportions of AgNP and CuNP were mixed thoroughly to produce the bimetallic Ag-CuNPs and were stored at 4 °C until further experimental analysis.

Characterization of Ag, Cu and Ag-Cu bimetallic nanoparticles

UV-Visible spectral analysis of monometallic nanoparticles (AgNP and CuNP) and bimetallic Ag-CuNPs was performed using spectrophotometer (model LABINDIA UV-3000+ spectrometer) to record the surface Plasmon resonanace (SPR), dependent on shape, size distribution of nanoparticles. Fourier transform infrared (FTIR) spectra are used to study the surface adsorption of functional groups on nanoparticles and gives information on the physical state and chemical composition of the whole sample. The bio-reduction compounds that are responsible for the synthesis of NPs were determined using Fourier Transform Infrared spectroscopy (FTIR). The FTIR spectra were collected from 16 scans per sample at a resolution of 4 cm^{-1} at a range of 400-4000cm^{-1} using Bruker optics, Germany (model; TENSOR 27). Scanning electron microscope (SEM**)** (Hitachi 500 with 15.0 kV with 5.00µm) is used to study the shape and size. Size and shape of *Achyranthes aspera* synthesised NPs studied. The crystalline structure of nanoparticles was determined using X-ray diffraction (XRD) spectroscopy (Bruker AXS D-8 Advance power X-ray diffractometer (Shimadzu, Japan) operated at a voltage of 40 kV and current of 30 mA using equipped with Cu Kα radiation (λ= 1.5406 Å) as radiation source at a current of 30 mA, with an application of 45kV tension. The scan parameter was set at a scan rate 1.2 per minute and 2θ ranges between 0° and 80°.

Antiproliferative activity

Cell culture and maintenance

The MCF-7 cells (human breast adenocarcinoma cells) were obtained from the repository cell line centre of the National Centre for Cell Science (NCCS), Pune, India. They have been maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-Glutamine, 0.1 mM (milli Molar) nonessential amino acid, penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a humidified environment with 5% CO_2[21].

MTT assay

MTT Assay, which is the colourimetric (3-(4,5 dimethylthiazole-2yl-diphenyl tetrazolium bromide) reduction assay used to assess cell viability of cells in increasing doses. To determine whether there were viable cells in the cell suspension, the cells were trypsinized and subjected to the tryphan blue assay. The cells were counted using a hemocytometer, and cells is seeded in of 96-well plate culture medium at a density of 5.0×10^3 cells/well, and then incubated for the entire night at 37°C. Following incubation, add 100 µL of fresh media containing extract and nanoparticles at varying concentrations (5, 10, 25, 50, and 100 µg/mL) to each well after existing medium removed. Cisplatin was employed as a positive standard, and only PBS was applied to the control wells devoid of extracts. After 48 hours, discard the drug solution and fill each well with fresh medium with 10µL MTT solution (5 mg/mL). The plates were

then incubated for 3 hours at 37°C. The cells with metabolically active mitochondria reduce the MTT salt to purple chromophore formazan crystals, which results in precipitates at the conclusion of the incubation period. Using a microplate reader, the optical density of solubilized crystals in 100 µL DMSO was determined at 570 nm. The formula below was used to determine the percentage of cell viability. A control well containing media without nanoparticles at the specified concentrations was included. The percentage of inhibition was calculated using a specific formula, and the concentration of nanoparticles required to inhibit cell growth by 50% (IC_{50}) values was determined[22].

$$\% \text{ Cell viability} = \frac{A_t - A_b}{A_c - A_b} \times 100$$

Where, A_t = Absorbance of test compound, A_b = Absorbance of blank and A_c = Absorbance of control.

Statistical analysis

The experimental data were expressed as mean ± standard deviation of values in triplicate from three different experiments. The significance of difference among the various treated groups and positive and negative control groups was analysed by means of one-way analysis of variance (ANOVA), and the level of significance was set at $P < 0.05$.

Results and Discussion

Achyranthes aspera, a perennial herb belongs to the family the Amaranthaceae family, possesses many therapeutic activities, like antioxidant, anticancer[29], antimicrobial[31,32], antifertility[32], antipyretic33, hypolipidemic[33] hepatoprotective activity[33], anti-inflammatory[34], wound healing[34]. A lot of research was conducted on *Achyranthes aspera* (stem, roots, and leaves) extracts which have various therapeutic activities and pharmaceutical applications [35,36]. The phytochemical analysis on the *Achyranthes aspera* confirms the presence of alkaloids, flavanoids, phenols, saponins and tannins[37]. These bio-active compounds of *A. aspera* are reduction of silver nanoparticles from Ag^+ to Ag^o and Copper nanoparticles from Cu^{2+} to CuNP and together forming Ag-Cu bimetallic nanparticles.

Characterisation

UV-visible absorption study

The UV-visible absorbance of *A. aspera* stem extract with Ag^+ and Cu^{2+} ions was determined by Surface Plasmon resonance peak at 426 nm and 532 nm which confirms the formation of AgNPs[23] and CuNPs[25] respectively. When compared among the monometallic nanoparticles, the higher wavelengths, copper is a weak absorber. In contrast to the equal ratio mixing of the sample, AgNPs and CuNPs resulted in the bimetallic Ag-Cu nanoparticle and its SPR revealed the two bands at 400nm and 540nm[24](Fig. 1).

FTIR spectral analysis

Using Fourier transform infrared spectrometer analysis, the functional groups of the phytomoieties found in the *A. aspera* aqueous extract that were in charge of stabilising and reducing the Ag and Cu metal ions. Numerous biomolecules, such as alkaloids, flavonoids, terpenes, tannins, glycosides, steroids, reducing sugars, and saponins, are present in *Achyranthes aspera* aqueous stem extract[35]. The FTIR spectrum of AgNPs synthesised from *Achyranthes aspera* showed the absorption peaks at at 1045.45 cm^{-1} could be due to C-N stretch stretching mode of amine. The absorption peak at 1631.83 and 1641.48 cm^{-1} may due to alkenyl or aromatic C=C stretch. The absorption bands at 2901.04 and 2982.05 cm^{-1} are assigned to C-H stretch of alkanes. The absorption bands at 3452.15 cm^{-1} are assigned to phenolic[26] O-H stretch (Fig. 2A). The FTIR spectrum of CuNPs synthesized from *Achyranthes aspera* showed the absorption peaks at 1049.31 cm^{-1} is due to C-N stretch of amine. The absorption peak at 1629.90 and 1635.69 cm^{-1} may due to alkenyl or aromatic C=C stretch. The absorption bands at 2341.66 and 2460.95 cm^{-1} might be due to amine C=H stretch[27]. The absorption bands at 3452.15 cm^{-1} are assigned to phenolic O-H stretch (Fig. 2B).

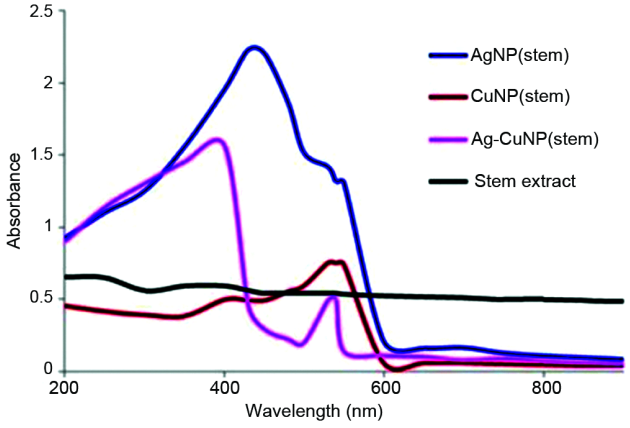

Fig. 1 — UV-Visible spectrophotometer spectrum of silver nanoparticles, Copper nanoparticles and bimetallic silver-copper nanoparticles synthesised with *Achyranthes aspera* plant stem extract.

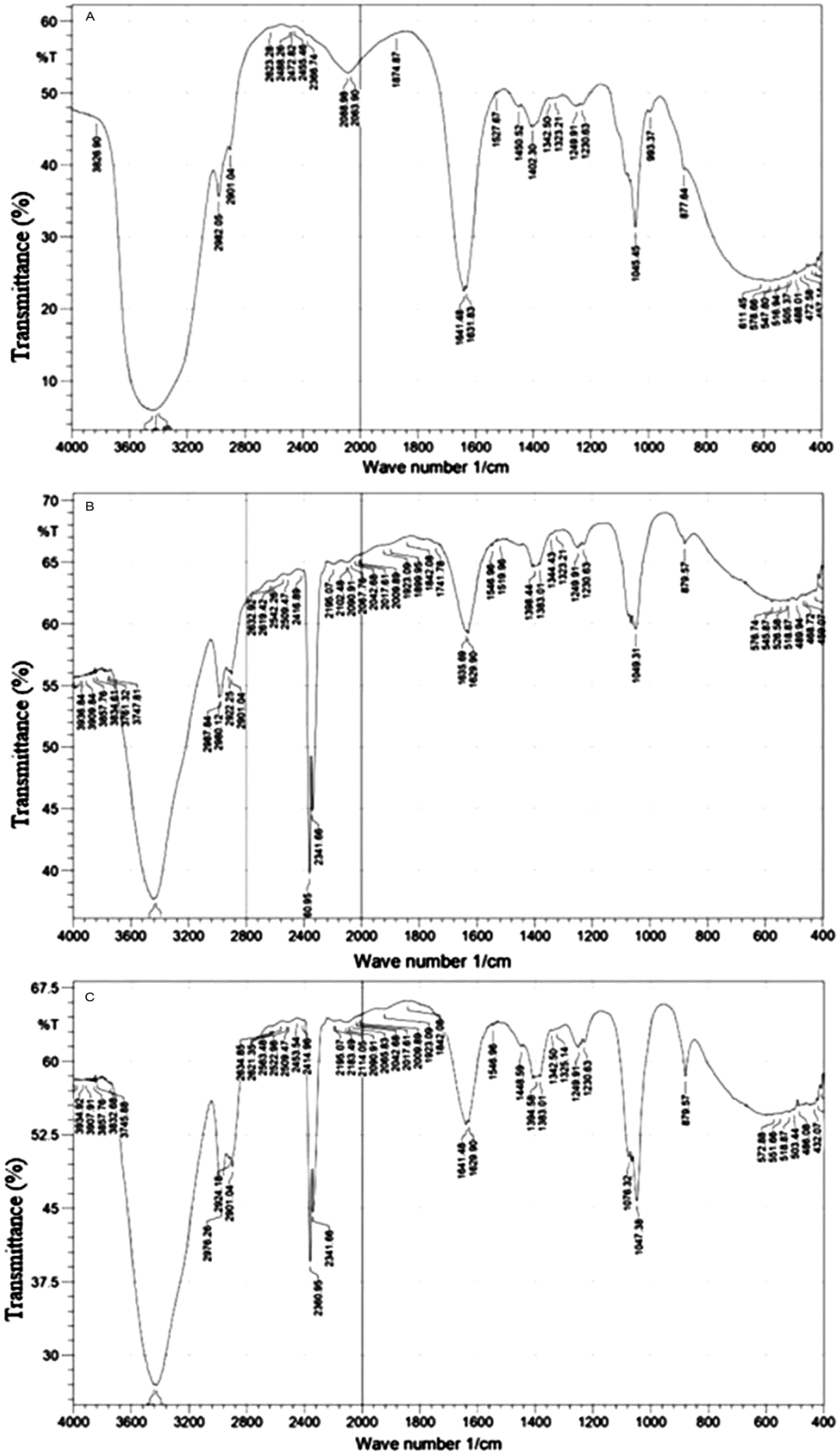

Fig. 2 — FTIR spectrum of silver nanoparticles (A); Copper nanoparticles (B); Bimetallic silver-copper (C) nanoparticles synthesised from *Achyranthes aspera* stem extract.

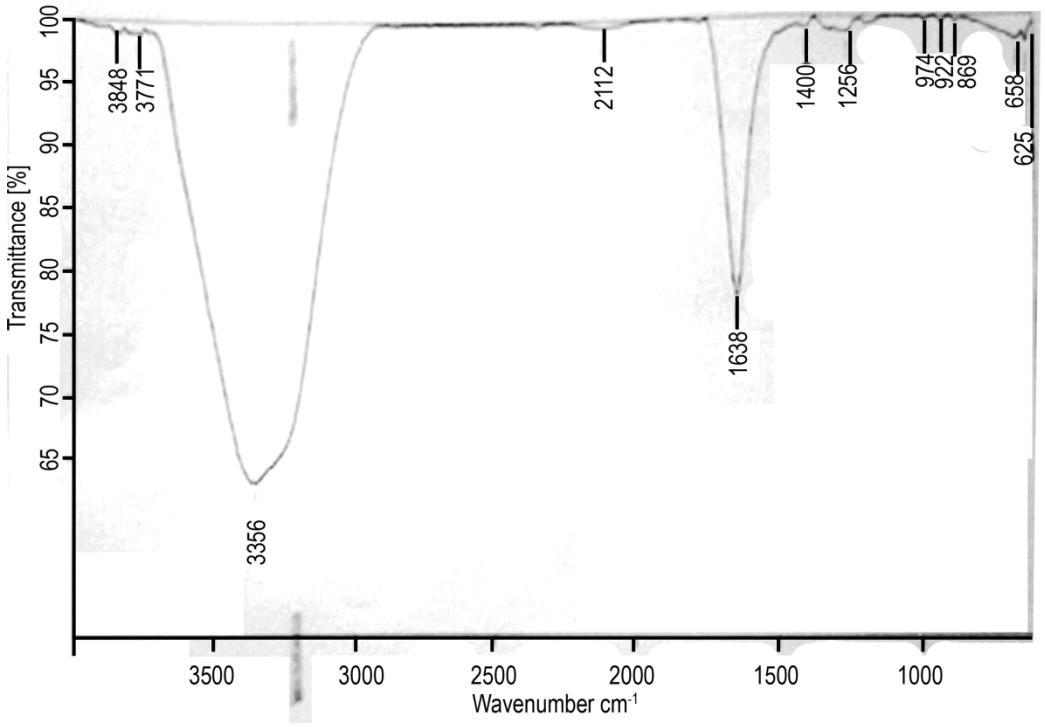

Fig. 3 — FTIR spectrum of stem aqueous extracts of *Achyranthes aspera*.

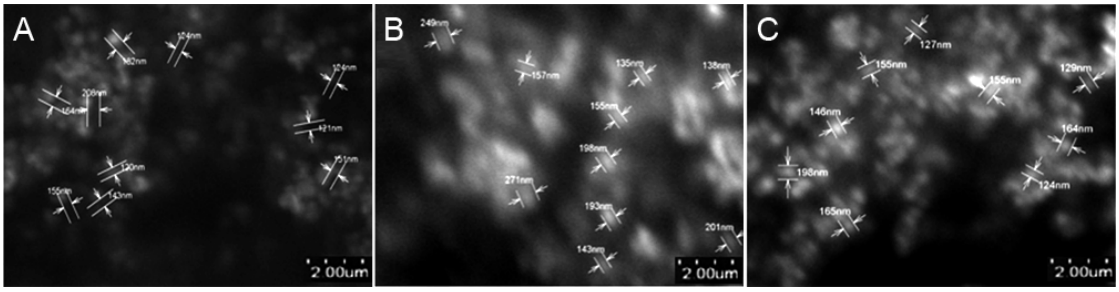

Fig. 4 — SEM images of AgNPs (A); CuNPs (B); Ag-CuNPs (C) synthesised using *Achyranthes aspera* stem extract.

The FTIR spectrum of bimetallic Ag-CuNPs synthesised from *Achyranthes aspera* showed the absorption peaks at 1047.38 cm^{-1} and 1076.32 cm^{-1} could be due to C-H stretching mode of amine. The absorption peak at 1629.90 cm^{-1} and 1641.48 cm^{-1} may due to alkenyl or aromatic C=C stretch[28]. The absorption bands at 2341.66 cm^{-1}, 2380.95 cm^{-1} and 2901.04 cm^{-1} are assigned to amine C=H stretch. The peak at 3452.15cm^{-1} corresponds to phenolic O-H stretch (Fig. 2C). The FTIR spectrum of *Achyranthes aspera* aqueous stem extract illustrated in (Fig. 3), revealed strong peaks at 1400 cm^{-1} and 1638cm^{-1} indicates the presence of O-H inorganic carbonate stretch bond and C=C stretching alkene respectively, while 2112 cm^{-1} represents C≡C stretching of alkynes[29]. The absorption bands at 3356cm^{-1} correspond to the presence of phenol –OH group. When the FTIR spectra reports compared the nanoparticles with the extract, which indicates the marginal shift in the spectra and indicates the presence of the phytochemicals which acts a reducing and stabilising agent, and aid in the formation of nanoparticles AgNPs, CuNPs and Bimetallic Ag-CuNPs.

Surface morphology analysis

The surface morphology of nanoparticles was determined using scanning electron microscopy (SEM). The size of the emerged AgNPs synthesised from the stem aqueous extract of *A.aspera* ranges from 120 to 208 nm (Fig. 4A) and is found to be round and small spherical in shape, while CuNPs showed sizes of ranging from 135 to 249 nm (Fig. 4B)

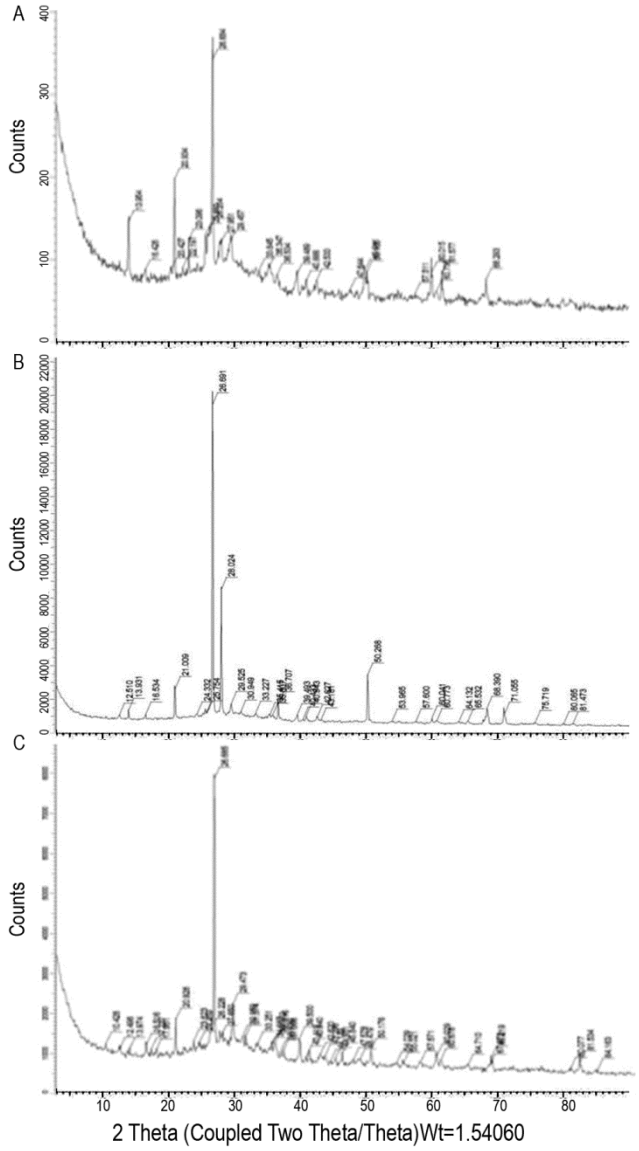

Fig. 5 — XRD pattern of AgNPs (A); CuNPs (B); Bimetallic Ag-CuNPs (C) synthesised using *Achyranthes aspera* stem extract.

with rod shapes. The stem bimetallic Ag-CuNPs are found to be in the ranges of 124 to 198 nm (Fig. 4C) with rod and round shapes.

X-ray diffraction analysis

The X-ray diffraction (XRD) pattern of the biosynthesised *A.aspera* stem AgNPs[30] shows the following diffraction peaks prominently of 2θ at 13.95, 20.93, 23.09, 26.68, 60.01 (Fig. 5A). These major peaks in the spectrum, corresponds to the (111), (211), (202), (311) and (551) planes, respectively, which reflect the patterns of the face-centered cubic (fcc) and crystalline structure of the biosynthesised AgNPs. CuNPs synthesised from *Aspera* stem caused an XRD diffraction pattern with diffraction angles (2θ) at 21.01, 26.69, and 50.26 (Fig. 5B) and corresponding crystal planes of (111), (200), and (400) for copper nanoparticles. The bimetallic Ag-CuNPs showed the prominent peaks of 2θ at 16.51, 20.92, 26.68, 39.5(Fig. 5C) and corresponding crystal planes (111), (200), (220) and (400)[31]. Various reducing agents present in the extract contribute to the stabilisation of nanoparticles and provide them with a crystalline structure, a phenomenon that has been extensively studied in various nanoparticle synthesis.

***In vitro* antiproliferative activity against MCF-7 cancer cell lines**

MTT assay

MTT Assay is a colourimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The assay depends both on the number of cells present and, on the assumption, that dead cells or their products do not reduce tetrazolium. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, dark purple colored formazan crystals. The cells are then solubilised with a DMSO and the released, solubilised formazan reagent is measured spectrophotometrically at 570 nm.

Experimental analysis

To demonstrate the antiproliferative activity of the green synthesised *A. aspera* nanoparticles, different doses of nanoparticles (AgNps, CuNPs, Ag-CuNPs) were added to the MCF-7 cell lines; where, cisplatin used as standard drug. This study employed the MTT assay to assess the anticancer activity of NPs on MCF-7 cancer cell lines. To confirm whether the treatment reduced cancer cell viability, samples with concentrations of 5, 10, 25, 50, and 100 µg/mL were utilized. The experimental results demonstrated that higher concentrations of NPs reduced the cell viability. Among the extract of 100 µg/mL concentration of AgNPs, CuNPs, Ag-CuNPs synthesised from aqueous extract of *A.aspera* the bimetallic Ag-CuNPs showed the *less* cell viability against MCF-7 cell lines. (Fig. 6). Statistically significant difference was found at all the concentrations; however, highest significance was observed at 100 µg/mL bimetallic Ag-CuNPs (P<0.001)

Determination of IC_{50} values

The IC_{50} values (concentration required to inhibit 50% of cell viability)[31] were calculated from the graph using the equation $Y = mX$ and the linear

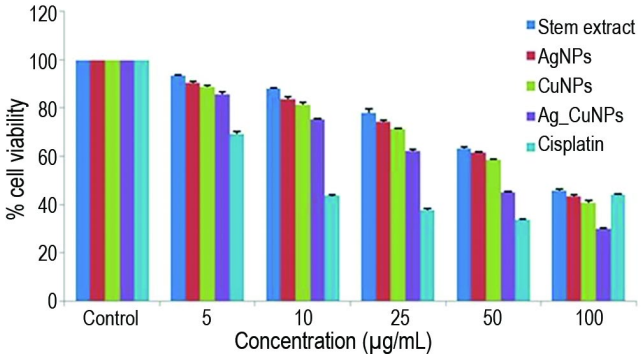

Fig. 6 — Graph on dose-dependent effect of concentration of AgNPs, CuNPs, bimetallic Ag-CuNPs comparison to stem extract and cisplatin on cell viability against MCF-7 cell lines determined by MTT assay. [Data were expressed as mean ± standard deviation of values in triplicate from three different experiments. The significance of difference among the various treated groups and positive and negative control groups was analysed by means of one-way analysis of variance (ANOVA), and the level of significance was set at $P < 0.05$]

Table 1 — IC_{50} value of stem extracts, AgNPs, CuNPs, Ag-CuNPs synthesized from aqueous stem extract of *Achyranthes aspera* on MCF-7 cell lines by MTT assay

Extracts	IC_{50} values
Stem extract	86.11±0.612
AgNPs	81.40±0.823
CuNPs	75.66±0.514
Ag- CuNPs	55.28±0.517
Cisplantin	7.17±0.141

Values are expressed as Mean±SD

regression coefficient. The bimetallic silver-copper nanoparticles derived from *Achyranthes aspera* stem extract exhibited anti-cancer activity against MCF-7 cells, with an IC_{50} of 55.28±0.517µg/mL; copper nanoparticles derived from *Achyranthes aspera* stem extract demonstrated an IC_{50} of 75.66±0.514µg/mL, while silver nanoparticles exhibited an IC_{50} of 81.40±0.823µg/mL against MCF-7 cells. The stem extract of *Achyranthes aspera* demonstrated anti-cancer activity against MCF-7 cells, with an IC_{50} of 86.11±0.612µg/mL. The conventional drug compound cisplatin demonstrated anti-cancer activity against MCF-7 cells, with an IC_{50} of 7.17±0.141µg/mL. (Table 1, Fig. 7). The assay concluded that bimetallic silver-copper nanoparticles have limited the cell viability of MCF-7 cancer cells compared to other synthesized nanoparticles[38,39]. Similarly, the morphological changes of MCF-7 cells were observed using an inverted microscope. Treatment of MCF-7 cells were performed with (5 µg, 10 µg, 25 µg, 50 µg, 100 µg) concentration of Stem extract synthesised

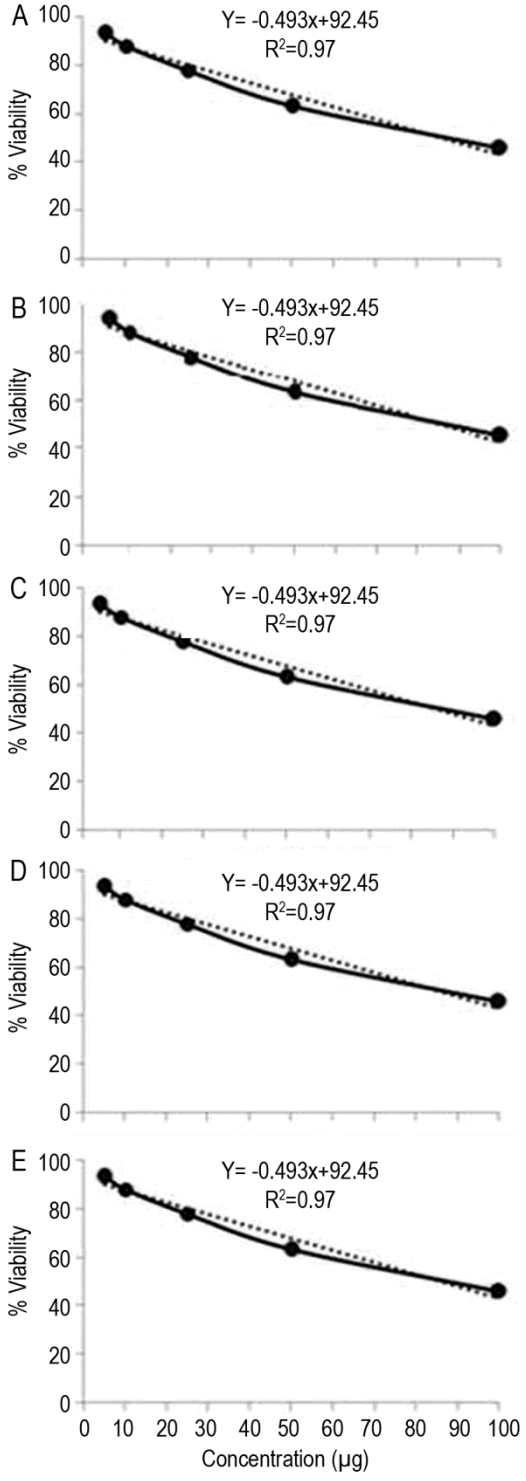

Fig. 7 — Evaluation of cytotoxicity of (A) Stem extract of *A. aspera*; (B) AgNP synthesised from aqueous stem extract of *A. aspera*; (C) CuNP synthesised from aqueous stem extract of *A. aspera*; (D) Ag-CuNP synthesised from aqueous stem extract of *A. aspera.* (E) Cisplatin, on MCF-7 cells using MTT assay. The IC_{50} was calculated. [Values obtained from three separate experiments are shown (mean ± SD) and the nonlinear regression curve was added to plot, $R^2 = 0.972$]

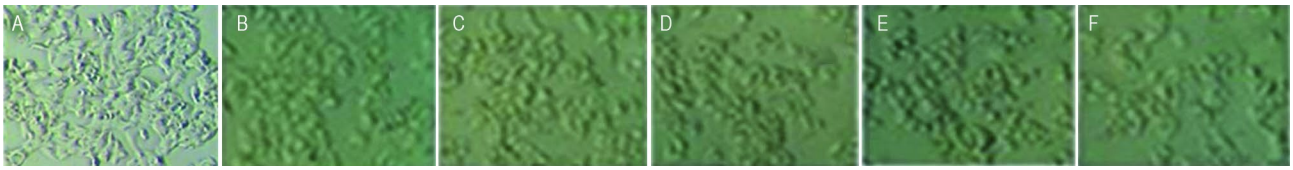

Fig. 8 — (A) Untreated cell and Bright-field inverted microscope images depicting the detrimental impact of Cisplatin (B) 5µg/mL, (C) 10 µg/mL, (D) 25 µg/mL, (E) 50 µg/mL, and (F) 100 µg/mL concentrations on the micro-morphology of MCF-7 cell lines.

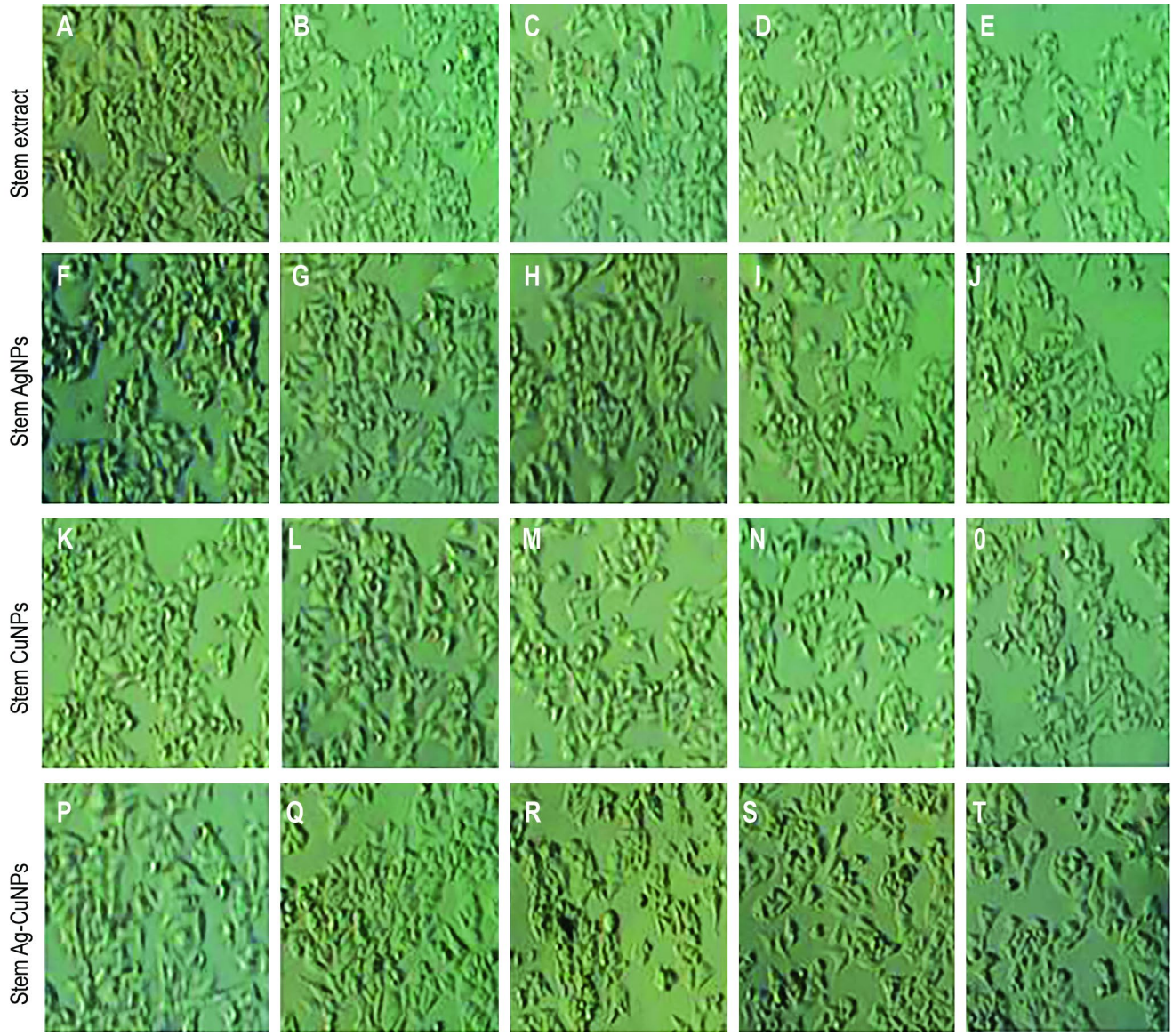

Fig. 9 — Bright-field inverted microscope images depicting the detrimental impact of Nanoparticles synthesised from aqueous stem extract of *Achyranthes aspera* (A,F,K) 5µg/mL; (B,G,L) 10 µg/mL; (C,H,M) 25 µg/mL; (D,I,N) 50 µg/mL, and (E,J,O) 100 µg/mL) concentrations on the viability of MCF-7 cell lines.

from *A.aspera*, AgNPs, CuNPs, bimetallic of Ag-CuNPs of stem *A.aspera* and standard drug Cisplatin. The study demonstrated that Ag-CuNPs adversely induced micro-morphological alterations compared to the other nanoparticle and cisplatin, which had an impact on the viability of the cells (Fig. 8 & 9). The observed outcomes were discovered to be consistent with MTT assay. Statistically significant difference was found at 100µg/mL concentration. The study revealed that at the higher concentration of

100 µg/mL, bimetallic of Ag-CuNPs of stem *A.aspera* showed the significant % cell inhibition compared to the stem extract, AgNPs and CuNPs of *A.aspera*.

Conclusion

The findings of the study reveal a sustainable method for synthesising AgNPs, CuNPs, and bimetallic Ag-CuNPs using aqueous stem extract of *Achyranthes aspera*. This method not only minimises environmental impact but also enhances the stability and efficacy of the nanoparticles, making them promising for various field applications. Consequently, it presents an alternative to traditional methods that often involve harmful chemicals. Compared to cisplatin, the biosynthesised AgNPs, CuNPs, and the bimetallic Ag-CuNPs exhibit enhanced anticancer activity with reduced cytotoxicity to normal cells. Thus, it is concluded that the green synthesised nanoparticles AgNPs, CuNPs, and bimetallic Ag-CuNPs are eco-friendly and cost-effective showing promising anticancer activity against MCF-7 cancer cell lines (*in vitro*). Among the synthesised nanoparticles, the bimetallic Ag-CuNPs demonstrated increased antiproliferative activity, attributed to the synergistic interaction between the two monometallic nanoparticles. Further research is needed to explore the mechanisms behind the enhanced anticancer properties of these bimetallic nanoparticles. Additionally, *in vivo* studies will be essential to evaluate their efficacy and safety in living organisms, paving the way for potential clinical applications.

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Conflict of interest

The authors declare no conflict of interest related to this study.

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